Practitioner's Docket No. MPI01-019P1RNM ***PATENT***

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Curtis, Rory A.J.		
Application No.:	10/074547	Group No.:	1647
Filed:	February 12, 2002	Examiner:	Hunnicutt, Rachel Kapust
For:	25466, A HUMAN TRANSPORTER FAMILY MEMBER AND USES THEREFOR		

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

I, Sunita Badola 310 Old Westford Road, Chelmsford, MA 01824, hereby declare and state:

1. I am currently employed by Millennium Pharmaceuticals, Inc. as a Manager II in the Transcriptional Profiling group and am trained as a Molecular biologist and Protein biochemist. I have been performing Taqman Real Time PCR analyses for six years. I manage the Real Time PCR core group and supervise the analysis functions of this group at Millennium. My curriculum vitae is attached as Exhibit A.

2. The above-identified patent application was filed on behalf of Millennium Pharmaceuticals, Inc., 40 Landsdowne Street, Cambridge, Massachusetts 02139, the Assignee of record. This patent application is a utility application which claims priority to U.S. Provisional Application Numbers 60/269,072 filed February 15, 2001.

CERTIFICATION UNDER 37 C.F.R. SECTIONS 1.8(a) and 1.10*

I hereby certify that, on the date shown below, this correspondence is being:

MAILING

☑ deposited with the United States Postal Service in an envelope addressed to the Mail Stop AF, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

37 C.F.R. SECTION 1.8(a) **37 C.F.R. SECTION 1.10***

☑ with sufficient postage as first class mail. ☐ as "Express Mail Post Office to Addressee" Mailing Label No.

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☐ transmitted by facsimile to the Patent and Trademark Office.

Signature

Sean Hunziker/Beverly Sotiropoulos
(type or print name of person certifying)

Date: NOVEMBER 30, 2004

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3. I am familiar with the application, the invention claimed therein and have reviewed the rejection of these claims under 35 U.S.C. §101, set forth in the Office Action mailed from the U.S. Patent and Trademark Office on September 30, 2004. The Examiner states that claims 1-7, 12 and 23-25 are not supported by either a specific and substantial asserted utility or a well established utility. Specifically, the Examiner states that the expression does not depend on any characteristics of the nucleic acid molecule itself and that further research to identify or reasonably confirm a "real world" context of use are not substantial utilities. The Examiner further alleges that the specification does not teach whether the tumor tissue cited for expression results was malignant or benign and believed it important to know whether cell lines or biopsies were used. The Examiner further takes issue with the statements of relative levels of expression (high, medium or low) as providing insufficient data to enable one of skill in the art to diagnose any particular cancer. I disagree with the Examiner for the reasons set forth in the following paragraphs.

4. I devised the probe and primers for and performed the Taqman® quantitative PCR transcriptional profiling studies on the 25466 gene (SEQ ID NO:1) for the Applicant. I have enclosed Exhibit B, a copy of SEQ ID NO:1 from the sequence listing of the present application. Within this copy, I inserted the sequences for the primers and probes I devised and used for the studies described in the Gene Expression Analysis Example in the specification. One can see on page 3 of Exhibit B, that the Taqman reagents amplify nucleotides 733-1813 of SEQ ID NO:1, encoding amino acids 429-455 of the 25466 polypeptide (SEQ ID NO:2). This map of the Taqman reagents on SEQ ID NO:1 makes it clear that the expression data are specific to the nucleic acid molecule claimed in the application, as stated in the specification at paragraph [00368]. Detection of expression of 25466 by this method requires annealing of all three reagents (F1, R1 and P1) to the transcript and does depend on the characteristics of SEQ ID NO:1.

5. The tumor samples used in the Gene Expression Analysis Example were transcripts obtained from biopsies of tumors of organs and tissues including ovary, lung, colon, prostate and breast. The ovary tumor sample was a pool of different patient biopsy from a late stage epithelial tumor, stage III/IV; the lung tumor sample was a pool of different patient biopsy from Non-small cell carcinoma and squamous cell carcinoma with >80% tumor; the colon tumor sample was a pool of different patient biopsy from adenocarcinoma, with >80% tumor ; the prostate tumor was a pool of different patient biopsy from a adenocarcinoma with >70% tumor, the breast tumor sample was pool of biopsy from a Infiltrating Ductal Carcinoma with >90% tumor. All these tissues were evaluated by Histopathology by a board certified MD Pathologist who confirmed the disease stage prior to use in Taqman panels. All these biopsies are obtained at a metastatic stage of the cancer. None of the tumors used in the assay are benign. The nucleic acid was prepared by standard RNA isolation methods from a frozen tissue block, e.g. homogenization of the tissue and isolating RNA and treating with DNAse I, then the nucleic acid was precipitated, as described in paragraph [00366] of the specification. As can be seen by this information, the samples used in the analysis were taken directly from tumor tissue, so the differential expression observed is indicative of actual conditions in the tumor. The caveat cited by the Examiner regarding differential gene expression by explanted tumors relative to cell lines as illustrated in Bover et al. has been considered, but does not apply to the present studies and conclusions. The differential expression of 25466 is seen in actual cancerous tissue and a comparison with normal tissue will enable one skilled in the art to recognize 25466 expression as a diagnostic marker for cellular proliferative and/or differentiative disorders of these tissues.

6. The Examiner took issue with Applicant's use of statements of relative (high, medium, low, trace), rather than absolute (present or absent) expression levels to determine diagnostic utility of 25466 expression analysis. For a standard for diagnostic utility, the Examiner refers to a statement about a receptor present in melanoma cells and absent in normal skin. However, that statement makes no

reference to how this presence is determined. The Taqman quantitative PCR method used in the present Gene Expression Analysis is a highly quantitative and sensitive method where minute amounts of mRNA are detected in a sample. An amount detected by this method as "trace," as described for ovary tumor, normal lung and normal colon, likely would be undetectable by another method such as in situ hybridization, or northern blot. In the present example, the actual multiple difference in the expression of 25466 among the pairs of samples is many-fold. The following table illustrates this point with actual quantitative values from the studies:

Sample	relative expression level described in paragraph [00370]]	actual expression	fold difference between paired samples
normal ovary	high	6.0452	21
ovary tumor	trace	0.2883	
normal lung	trace	0.0647	32
lung tumor	medium	2.0788	
normal colon	trace	0.0602	27
colon tumor	medium	1.6424	
normal prostate	low	0.9698	2.8
prostate tumor	medium	2.7336	
normal breast	low	1.334	2.5
breast tumor	medium	3.3888	

One can see from this table that some pairs of normal and tumor tissues have a many-fold difference in expression levels. This difference is reflected in the relative terms when a result is "high" or "medium" *versus* "trace" in the pair. Paired differences in 25466 expression levels of this magnitude are significant and one skilled in the art would be able to interpret this difference as a diagnostic indicator of a cancerous condition in ovary, lung and colon. The results when the relative terms describe "medium" *versus* "low" do not have as many-fold of a difference in expression levels. One skilled in the art would still recognize this numerical difference as significant, but may be less confident about 25466 expression in prostate and breast than in the sample pairs with larger relative differences in expression. In conclusion, the sensitivity of the Taqman method for detection of expression led to the relative terms for levels of expression, but those terms are indicative of levels one skilled in the art can rely upon for diagnostic interpretation of the presence of a cellular proliferative and/or differentiative disorder.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Sunita Badola

11/29/2006

Date

Docket No. MPI01-019P1RNM

U.S. Serial No. 10/1074,547, Filed February 12, 2002

Exhibits A, B, C1 and C2
Accompanying Response after Final Rejection
and Declaration under 37 C.F.R. § 1.132
to Office Action Dated September 30, 2004

Exhibit A	Curriculum Vitae of Sunita Badola
Exhibit B	SEQ ID NO:1 from the Sequence Listing of the Present Application
Exhibit C1	Brown, T., Unit 2.10, "Hybridization Analysis of DNA Blots," *Current Protocols of Molecular Biology*, (1993)
Exhibit C2	Brown, T., Unit 6.3, "Using DNA Fragments as Probes," *Current Protocols of Molecular Biology*, (1993)

SUNITA BADOLA
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(H) 978-250-1855
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EDUCATION

Bioinformatics Essentials certificate 2003 – Northeastern University, Boston, MA
M.S. in **Biochemistry**, **May 1998**, Case Western Reserve University, Cleveland, OH.
M.S. in **Biotechnology**, **June 1993**, Indian Institute of Technology (IIT), Roorkee, India.
Bachelor of Science in **Chemistry, Botany** and **Zoology**, **June 1991**.
H.N.B. Garhwal University, Dehradun, India

WORK EXPERIENCE

Millennium Pharmaceuticals **Nov 2004- Present**
45 Sidney Street,
Cambridge, MA 02139

Manager II Transcriptional Profiling

- Manage the Real Time PCR Core group (3 individuals) within Transcriptional Profiling such that Inflammation and Oncology group within MPI receive sufficient Expression profiling analysis to meet their respective goals

Research Investigator **Feb 2002- Nov 2004**

- Manage the Real Time PCR Core group (3 individuals)
- Successfully evaluated and implemented the outsourcing of Taqman reagent design and QC to Epoch Biosciences. Coordinate and Monitor the reagent quality and Turn around Time on a weekly basis with Epoch Biosciences.
- Established relationships with Scientists from MCB, Oncology and Inflammation groups to evaluate expression profile projects and manage their expectations
- Currently working on Taqman data analysis of Protease and protease inhibitor interactions
- Generated Spotfire Taqman Data analysis reports for scientists to easily evaluate 1000's of genes with given disease specific criteria eg Evaluated Kinome analysis and oncology target selection reports for discovery efforts.
- Established relationships with Informatics group to embark on Taqman-Paris integration Project
- Worked closely with DSD Toxicity gene expression group to evaluate array of genes in predicting toxicity in Human and Rat hepatocyte in vitro studies.

Senior Research Associate **Feb 14,2000- Feb14, 2002**

- Managed a Taqman PhaseI group (4-8 people) to evaluate expression profiles

- Coordinated the TaqMan Gene Initiative so that the expression of 1000 proprietary Millennium genes are profiled on the Molecular Pathology Phase 1 TaqMan panel within 8 months
- Participated in writing Taqman SOP and trained people within and outside of Mol. Path. in conducting TaqMan experiments by giving consultations, workshops and seminars.
- Coordinated the development and implementation of chemical, informatic, and automation technologies by outside groups (ex. Oligo core, Trace, Process Tech, IT, etc.), to increase the quality and efficiency of Phase 1 TaqMan expression profiling with Molecular Pathology

Scriptgen Pharmaceuticals, Inc., **Jan 1, 1999 – Feb12,2000**
610 Lincoln street,
Waltham, MA-01254

Associate Scientist II :

- Responsible for the Hepatitis B Core project by coordinating the efforts from HTS, protein purification and developing antiviral secondary assays to test the hit compounds.
- Lead the development of Taqman methodology (quantitative PCR by using the fluorescent probes) for analysis of HCV, BVDV and WHV containing samples.
- Responsible for developing and analyzing the samples from Antiviral efficacy studies for both Hepatitis B virus and Hepatitis C Virus therapeutics at Scriptgen, which will enhance the in-house development capabilities.
- Responsible for designing, cloning, and subcloning the BVDV-HCV chimera that will be used for testing the antiviral compounds in the cell based assays.

Scriptgen Pharmaceuticals, Inc.,
610 Lincoln street,
Waltham, MA-01254

Associate Scientist I : **March 15,1998- Dec 1998**

- Participated actively in new Antiviral target identification, selection and prioritizing the protein targets.
- In conjunction with the project supervisor, devised a working science plan for the discovery phase for the target.
- Actively participated in the laboratory as well as coordinated the efforts of protein purification, high throughput screening, and antiviral technology development
- Coordinated a journal club on topics of interest to the Antiviral group.

Case Western Reserve University, **August 1995- May 1998**
Department of Biochemistry, Cleveland, OH.

Research Assistant:

- An extensive analysis of poly(P) glucokinase enzyme from *Mycobacterium tuberculosis* was performed to understand the structure-function of polyphosphate dependent enzyme by site directed mutagenesis. Methods for the analysis included initial velocity study, product inhibition analysis, dead end inhibition study along with the product distribution analysis. This project was supported by National Institutes of Health (GM 29569).

Case Western Reserve University, **December1994- August 1995**
Department of Biochemistry, Cleveland, OH.

Research Assistant:

- Involved in the study of regulation of C-reactive protein at transcriptional level.
- Investigated the sequence in promoter region of CRP, activated by several transcriptional factors, designed and constructed block mutants, identified by DNA sequencing, effect of mutation identified by transfection in Hep3B cells and treating with different cytokines, analysed by CAT assays.

G. B. Pant Hospital, **December 1993- September 1994**
Department of Gastroenterology, New Delhi, India

Junior research Fellow:

- Responsible for creating a literature profile for HBV and HCV.
- Involved in the study of prevalence and profile of chronic liver disease due to hepatitis B and hepatitis C virus.
- Detected HBV by ELISA, pathological assessment of cirrhosis by silver staining.

University of Madras, **May 1992-Aug1992**
Center for Advanced Studies in Botany, Madras, India.

Junior research Assistant:

- Involved in cloning of Pectate lyase gene in Pseudomonas.
- Learnt application of recombinant DNA techniques.

BIOTECHNOLOGICAL SKILLS:

- Chromatographic methods (ion-exchange, affinity, gel filtration)
- Electrophoretic methods (Native, SDS-PAGE, Western Blot, Gel Shift analysis, Electro Chemi-luminescence)
- Spectroscopic techniques (fluorescence, Circular dichroism and UV-Vis)
- Enzyme assays and enzyme kinetics
- Screen Library, PCR, Cloning, subcloning, RT-PCR, DNA isolation, RNA isolation, restriction analysis, DNA electrophoresis, DNA sequencing, Southern hybridization

- Site directed mutagensesis
- Expression and purification of recombinant proteins in *E.coli*
- Tissue culture and mammalian culture (HUH-7 cells , HepG2 cells)
- Protein-Protein interaction, DNA-Protein interaction analysis
- Immunoprecipitation and immunoassay
- Direct ELISA and Sandwich ELISA
- Taqman ABI 7700/ABI 7900- Quantitative PCR analysis

Additional skills:

- Expert in Spotfire Data analysis, Clustal analysis, Sequencher, Primer Express Software
- Familiar with Winword, Excel, Power point, Gene runner, Grafit, McDNAsis, DNAstar, Primer express, Claris Draw, Rasmol, Kinemage, NCBI Blast, Expasy-Protein tools in PC and Mac system.
- Maintenance of laboratory equipments.

HONORS AND AWARDS

- Recipient of Certificate of merit in All India Senior Secondary Exams 1986
- MS (1991-1993) Scholarship awarded by Department of Biotechnology, Government of India, based on Nationally conducted exam
- Recipient of University Gold Medal in MS (Biotechnology 1991-1993)
- Recipient of Graduate research assistant scholarship (1995-1998), Case Western Reserve University, Cleveland, OH
- Awarded 'Bayer Star of the month' in July 2000 working in Bayer collaboration with Millennium Pharmaceuticals.
- Recipient of 'Outstanding contributor Award of the year 2000' by Millennium Pharmaceuticals.
- Invited as a Speaker in a Real Time Seminar series by Pyrosequencing in September 2003

EXHIBIT B to Accompany Response after Final Rejection and Declaration under 37 CFR § 1.132 in U.S. Serial No.10/074,547 to Office Action Dated September 30, 2004

Locations of Taqman® Primers and Probes on SEQ ID NO:1 (25466) for 10/074,547

<210> 1
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<212> DNA
<213> homo sapiens

<220>
<221> CDS
<222> (449)...(1981)

<400> 1

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aaggaagccc aggttaggtt tgggcacctc caaacgcacc cgttttaaag ccacctggac 180
tgaggcgtcg agctttcagc tccaccaaac gctcacctgg cctggcagcg agcggcggaa 240
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cgcgtctcca ggatgatagg cgacattgca acaaatctct acacccagca gctcaggggg 360
ctccaagcag agcagcaagt tcgaggatcc gggcgtggag ccgagtgagg ccgcagccca 420
gcgggcctcg ggcgaaaaat cttggaaa atg tat acc agt cat gaa gat att    472
                            Met Tyr Thr Ser His Glu Asp Ile
                             1               5

ggg tat gat ttt gaa gat ggc ccc aaa gac aaa aag aca ctg aag ccc   520
Gly Tyr Asp Phe Glu Asp Gly Pro Lys Asp Lys Lys Thr Leu Lys Pro
     10                  15                  20

cac cca aac att gat ggc gga tgg gct tgg atg atg gtg ctc tcc tct   568
His Pro Asn Ile Asp Gly Gly Trp Ala Trp Met Met Val Leu Ser Ser
 25                  30                  35                  40

ttc ttt gtg cac atc ctc atc atg ggc tcc cag atg gcc ctg ggt gtc   616
Phe Phe Val His Ile Leu Ile Met Gly Ser Gln Met Ala Leu Gly Val
                 45                  50                  55

ctc aac gtg gaa tgg ctg gaa gaa ttc cac cag agc cgc ggc ctg acc   664
Leu Asn Val Glu Trp Leu Glu Glu Phe His Gln Ser Arg Gly Leu Thr
             60                  65                  70

gcc tgg gtc agc tcc ctc agc atg ggc atc acc ttg ata gtg ggc cct   712
Ala Trp Val Ser Ser Leu Ser Met Gly Ile Thr Leu Ile Val Gly Pro
         75                  80                  85

ttc atc ggc ttg ttc att aac acc tgt ggg tgc cgc cag act gcg atc   760
Phe Ile Gly Leu Phe Ile Asn Thr Cys Gly Cys Arg Gln Thr Ala Ile
     90                  95                  100

att gga ggg ctc gtc aac tcc ctg ggc tgg gtg ttg agt gcc tat gct   808
Ile Gly Gly Leu Val Asn Ser Leu Gly Trp Val Leu Ser Ala Tyr Ala
105                 110                 115                 120

gca aac gtg cat tat ctc ttc att act ttt gga gtc gca gct ggc ctg   856
Ala Asn Val His Tyr Leu Phe Ile Thr Phe Gly Val Ala Ala Gly Leu
                125                 130                 135

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        155                 160                 165

gga ttc ggt acg ttc cta atg act gtg ctg ctg aag tac ctg tgc gca   1000
Gly Phe Gly Thr Phe Leu Met Thr Val Leu Leu Lys Tyr Leu Cys Ala
    170                 175                 180

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Glu Tyr Gly Trp Arg Asn Ala Met Leu Ile Gln Gly Ala Val Ser Leu
185                 190                 195                 200

aac ctg tgt gtt tgt ggg gcg ctc atg agg ccc ctc tct cct ggt aaa   1096
Asn Leu Cys Val Cys Gly Ala Leu Met Arg Pro Leu Ser Pro Gly Lys
                205                 210                 215

aac cca aac gac cca gga gag aaa gat gtg cgt ggc ctg cca gcg cac   1144
Asn Pro Asn Asp Pro Gly Glu Lys Asp Val Arg Gly Leu Pro Ala His
            220                 225                 230

tcc aca gaa tct gtg aag tca act gga cag cag gga aga aca gaa gag   1192
Ser Thr Glu Ser Val Lys Ser Thr Gly Gln Gln Gly Arg Thr Glu Glu
        235                 240                 245

aag gat ggt ggg ctc ggg aac gag gag acc ctc tgc gac ctg caa gcc   1240
Lys Asp Gly Gly Leu Gly Asn Glu Glu Thr Leu Cys Asp Leu Gln Ala
    250                 255                 260

cag gag tgc ccc gat cag gcc ggg cac agg aag aac atg tgt gcc ctc   1288
Gln Glu Cys Pro Asp Gln Ala Gly His Arg Lys Asn Met Cys Ala Leu
265                 270                 275                 280

cgg att ctg aag act gtc agc tgg ctc acc atg aga gtc agg aag ggc   1336
Arg Ile Leu Lys Thr Val Ser Trp Leu Thr Met Arg Val Arg Lys Gly
                285                 290                 295

ttc gag gac tgg tat tcg ggc tac ttt ggg aca gcc tct cta ttt aca   1384
Phe Glu Asp Trp Tyr Ser Gly Tyr Phe Gly Thr Ala Ser Leu Phe Thr
            300                 305                 310

aat cga atg ttt gta gcc ttt att ttc tgg gct ttg ttt gca tac agc   1432
Asn Arg Met Phe Val Ala Phe Ile Phe Trp Ala Leu Phe Ala Tyr Ser
        315                 320                 325

agc ttt gtc atc ccc ttc att cac ctc cca gaa atc gtc aat ttg tat   1480
Ser Phe Val Ile Pro Phe Ile His Leu Pro Glu Ile Val Asn Leu Tyr
    330                 335                 340

aac tta tcg gag caa aac gac gtt ttc cct ctg acg tca att ata gca   1528
Asn Leu Ser Glu Gln Asn Asp Val Phe Pro Leu Thr Ser Ile Ile Ala
345                 350                 355                 360

ata gtt cac atc ttt gga aaa gtg atc ctg ggc gtc ata gcc gac ttg   1576
Ile Val His Ile Phe Gly Lys Val Ile Leu Gly Val Ile Ala Asp Leu
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            380                 385                 390

gtc ctc agt att ttt att ctg ccg ttg atg cac acg tac gct ggc ctg   1672
Val Leu Ser Ile Phe Ile Leu Pro Leu Met His Thr Tyr Ala Gly Leu
        395                 400                 405

gcg gtc atc tgt gcg ctg ata ggg ttt tcc agt ggt tat ttc tcc cta   1720
Ala Val Ile Cys Ala Leu Ile Gly Phe Ser Ser Gly Tyr Phe Ser Leu
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                                           P1  C CTG GCC AAT
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Met Pro Val Val Thr Glu Asp Leu Val Gly Ile Glu His Leu Ala Asn
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gcc tac ggc atc atc atc tgt gct aat ggc atc tct gca ttg ctg gga   1816
Ala Tyr Gly Ile Ile Ile Cys Ala Asn Gly Ile Ser Ala Leu Leu Gly
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cca cct ttt gca ggg tgg atc tat gac atc acg caa aaa tat gat ttt   1864
Pro Pro Phe Ala Gly Trp Ile Tyr Asp Ile Thr Gln Lys Tyr Asp Phe
            460                 465                 470

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Ser Phe Tyr Ile Cys Gly Leu Leu Tyr Met Ile Gly Ile Leu Phe Leu
        475                 480                 485

ctt att cag ccg tgc att cga att ata gaa caa tcc aga aga aaa tac   1960
Leu Ile Gln Pro Cys Ile Arg Ile Ile Glu Gln Ser Arg Arg Lys Tyr
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acatctaagt tagacttgct cacgttcagt ttgtacagtt gtgtgttgac ttactatgtt 4111
ttgaaagtgg tgacttctac cgaatgagtg gaagttccca ttgtcaaaaa aaataaagac 4171
ctgcttgcag tattcatgtt gacaacagag taaaagagaa tactgtaaag aattactgca 4231
aatatttcct gtttatgtta tttgccgttg tttgaagata ttataaaggg ttaattgtat 4291
atttatatca tgtgctttat cgttttcccc tcatgtatcc aagtaatttt tatttacata 4351
caactaaata aatgttgtcc tctttgaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaggg 4411
gcggccgc                                                        4419
```

Hybridization Analysis of DNA Blots

The principle of hybridization analysis is that a single-stranded DNA or RNA molecule of defined sequence (the "probe") can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the "target"), with the stability of the hybrid depending on the extent of base pairing that occurs. Experimentally, the analysis is usually carried out with a probe that has been labeled and target DNA that has been immobilized on a membrane support. Hybridization analysis is sensitive and permits detection of single-copy genes in complex genomes. The technique has widespread applications in molecular biology.

The first stage in a hybridization experiment is to immobilize the denatured nucleic acids on a suitable solid support. Methods for achieving this with gel-fractionated and bulk DNA are described in *UNITS 2.9A & 2.9B*. The labeled probe is then applied in a solution that promotes hybridization. After a suitable incubation, the membrane is washed so that any nonspecifically bound probe is removed, leaving only probe that is base-paired to the target DNA. By controlling the stringency of the washing conditions, decisions can be made about whether to target sequences that are 100% complementary to the probe, or allow some mismatching so that sequences with lower degrees of similarity are also detected. The latter approach (heterologous probing) is used to study related sequences in a single or more than one genome.

Hybridization analysis was originally carried out with long (100 to 1000 bp), radioactively labeled DNA probes. Other types of probe (RNA, oligonucleotide) have more recently been introduced, as have nonradioactive labeling and detection strategies. In addition, improvements in understanding of the factors that influence hybrid stability and hybridization rate have led to a proliferation of reagents and protocols for hybridization analysis. Finding one's way through the maze can be a daunting task, especially as protocols that work well with one probe-target combination may not work so well if either member of the partnership is changed. The approach taken here is to present as the basic protocol an unsophisticated procedure for hybridization analysis with a radiolabeled DNA probe. Despite its lack of embellishments, the protocol gives acceptable results with Southern and dot blots on nitrocellulose and nylon (uncharged and charged) membranes. The alternate protocol describes a similar method for probing DNA blots with a radiolabeled RNA probe. A support protocol for stripping blots to ready them for reprobing is also provided.

Relevant units elsewhere in the manual include the following: *UNITS 3.18 & 3.19* describe the preparation of nonradioactive probes and their use in hybridization analysis; *UNIT 4.9* covers hybridization analysis of immobilized RNA; *UNIT 6.3* describes hybridization analysis of recombinant clone libraries; and *UNIT 6.4* explains how to use labeled oligonucleotides as hybridization probes.

These hybridization protocols should not be read in isolation. The commentary describes various modifications that can be introduced, including changes to prehybridization, hybridization, and wash solution formulations, and alterations to incubation times and conditions, the latter including a discussion of the wash conditions compatible with different degrees of stringency. The intention is provide the reader with sufficient data to make well-informed decisions about how to modify the basic and alternate protocols for specific applications.

CAUTION: Investigators should wear gloves for all procedures involving radioactivity and should be careful not to contaminate themselves and their clothing. When working with ^{32}P, investigators should frequently check themselves and the working area for

Contributed by Terry Brown

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Moderate stripping solution

200 mM Tris·Cl, pH 7.0
0.1× SSC (*APPENDIX 2*)
0.1% (w/v) SDS

Nucleotide mix

2.5 mM ATP
2.5 mM CTP
2.5 mM GTP
20 mM Tris·Cl, pH 7.5
Store at –20°C

COMMENTARY

Background Information

Hybridization between complementary polynucleotides was implicit in the Watson-Crick model for DNA structure and was initially exploited, via renaturation kinetics, as a means of studying genome complexity. In these early applications, the two hybridizing molecules were both in solution—an approach that is still utilized in "modern" techniques such nuclease protection transcript mapping (*UNITS 4.6 & 4.7*) and oligonucleotide-directed mutagenesis (Chapter 8). The innovative idea of immobilizing one hybridizing molecule on a solid support was first proposed by Denhardt (1966) and led to methods for identification of specific sequences in genomic DNA (dot blotting; *UNIT 2.9B*) and recombinant clones (*UNITS 6.3 & 6.4*). A second dimension was subsequently introduced by Southern (1975), who showed how DNA molecules contained in an electrophoresis gel could be transferred to a membrane (*UNIT 2.9A*), enabling genetic information relating to individual restriction fragments to be obtained by hybridization analysis.

Since the pioneering work of Denhardt and Southern, advances in membrane hybridization have been technical rather than conceptual. As reviewed by Dyson (1991), the detailed protocols have become more sophisticated, largely because of advances in understanding of the factors that influence hybrid stability and hybridization rate.

Hybrid stability is expressed as the melting temperature or T_m, which is the temperature at which the probe dissociates from the target DNA. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984):

$$T_m = 81.5°C + 16.6 (\log M) + 0.41(\%GC) - 0.61 (\%form) - {}^{500}/_{L}$$

and for RNA-DNA hybrids from the equation of Casey and Davidson (1977):

$$T_m = 79.8°C + 18.5(\log M) + 0.58(\%GC) - 11.8(\%GC)^2 - 0.56(\%form) - {}^{820}/_{L}$$

where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, %form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The practical considerations that arise from these two equations are summarized Table 2.10.2A.

The second important consideration in hybridization analysis is the rate at which the hybrid is formed. Hybrid formation cannot occur until complementary regions of the two molecules become aligned, which occurs purely by chance; however, once a short nucleating region of the duplex has formed, the remaining sequences base-pair relatively rapidly. The rate at which the probe "finds" the target, which is influenced by a number of factors (Table 2.10.2B), is therefore the limiting step in hybrid formation (Britten and Davidson, 1985). However, in practical terms hybridization rate is less important than hybrid stability, as in most protocols hybridization is allowed to proceed for so long that factors influencing rate become immaterial.

Critical Parameters

To be successful, a hybridization experiment must meet two criteria:

(1) *Sensitivity.* Sufficient probe DNA must anneal to the target to produce a detectable signal after autoradiography.

(2) *Specificity.* After the last wash, the probe must be attached only to the desired target sequence (or, with heterologous probing, family of sequences).

Parameters influencing these two criteria are considered in turn, followed by other miscellaneous factors that affect hybridization.

Factors influencing sensitivity

The sensitivity of hybridization analysis is determined by how many labeled probe molecules attach to the target DNA. The greater the number of labeled probe molecules that anneal, the greater the intensity of the hybridization signal seen after autoradiography.

Probe specific activity. Of the various factors that influence sensitivity, the one that most

frequently causes problems is the specific activity of the probe. Modern labeling procedures, whether nick translation, random oligonucleotide priming (*UNIT 3.5*), or in vitro RNA synthesis (alternate protocol), routinely provide probes with a specific activity of $>10^8$ dpm/μg. This is the minimum specific activity that should be used in hybridization analysis of genomic DNA, even if the target sequences are multicopy. If the specific activity is $<10^8$ dpm/μg, hybridization signals will be weak or possibly undetectable, and no amount of adjusting the hybridization conditions will compensate. If there is a problem in obtaining a specific activity of $>10^8$ dpm/μg, it is important to troubleshoot the labeling protocol before attempting to use the probe in hybridization analysis.

If the probe is labeled to 10^8 to 10^9 dpm/μg, it will be able to detect as little as 0.5 pg of target DNA. Exactly what this means depends on the size of the genome being studied and the copy number of the target sequence. For human genomic DNA, 0.5 pg of a single-copy sequence 500 bp in length corresponds to 3.3 μg of total DNA. This is therefore the minimum amount of human DNA that should be used in a dot blot or Southern transfer if a single-copy gene is being sought.

Amount of target DNA. There is, however, a second argument that dictates that rather more than 3.3 μg of DNA should be loaded with each dot or Southern blot. During hybridization, genuine target sequences (100% homologous to the probe) and heterologous target sequences (related but not identical to the probe) compete with one another, with the homologous reactions always predominant. Ideally this competition should be maintained until the end of the incubation period so that maximum discrimination is seen between homologous and heterologous signals. This occurs only if the membrane-bound DNA is in excess, so that target sequences are continually competing for the available probe (Anderson and Young, 1985). If the probe is in excess then the homologous reaction may reach completion (i.e., all genuine target sites become filled) before the end of the incubation, leaving a period when only

Table 2.10.2 Factors Influencing Hybrid Stability and Hybridization Rate[a]

Factor	Influence
A. Hybrid stability[b]	
Ionic strength	T_m increases 16.6°C for each 10-fold increase in monovalent cations between 0.01 and 0.40 M NaCl
Base composition	AT base pairs are less stable than GC base pairs in aqueous solutions containing NaCl
Destabilizing agents	Each 1% of formamide reduces the T_m by about 0.6°C for a DNA-DNA hybrid. 6 M urea reduces the T_m by about 30°C
Mismatched base pairs	T_m is reduced by 1°C for each 1% of mismatching
Duplex length	Negligible effect with probes >500 bp
B. Hybridization rate[b]	
Temperature	Maximum rate occurs at 20-25°C below T_m for DNA-DNA hybrids, 10-15°C below T_m for DNA-RNA hybrids
Ionic strength	Optimal hybridization rate at 1.5 M Na^+
Destabilizing agents	50% formamide has no effect, but higher or lower concentrations reduce the hybridization rate
Mismatched base pairs	Each 10% of mismatching reduces the hybridization rate by a factor of two
Duplex length	Hybridization rate is directly proportional to duplex length
Viscosity	Increased viscosity increases the rate of membrane hybridization; 10% dextran sulfate increases rate by factor of ten
Probe complexity	Repetitive sequences increase the hybridization rate
Base composition	Little effect
pH	Little effect between pH 5.0 and pH 9.0

[a]This table is based on Brown (1991) with permission from BIOS Scientific Publishers.

[b]There have been relatively few studies of the factors influencing membrane hybridization. In several instances extrapolations are made from what is known about solution hybridization. This is probably reliable for hybrid stability, less so for hybridization rate.

heterologous hybridization is occurring and during which discrimination between the homologous and heterologous signals becomes reduced. The problem is more significant with a double-stranded rather than a single-stranded probe, as with double-stranded probe reannealing between the two probe polynucleotides gradually reduces the effective probe concentration to such an extent that it always becomes limiting towards the end of the incubation.

In practical terms it is difficult to ensure that the membrane-bound DNA is in excess. The important factor is not just the absolute amount of DNA (which is dependent on the efficiency of immobilization and how many times the membrane has been reprobed) but also the proportion of the DNA that is composed of sequences (homologous and heterologous) able to hybridize to the probe. Rather than attempting complex calculations whose results may have factor-of-ten errors, it is advisable simply to blot as much DNA as possible: 10 μg is sufficient with most genomes. Assuming that the probe is labeled adequately and used at the correct concentration in the hybridization solution, a clear result will be obtained after autoradiography for a few hours with a simple genome (e.g., yeast DNA) or a few days with a more complex one (e.g., human DNA).

Labels other than ^{32}P. The discussion so far has assumed that the probe is labeled with ^{32}P. The lower emission energy of ^{35}S results in reduced sensitivity, and this isotope is in general unsuitable for hybridization analysis of genomic DNA. ^{35}S can be used only if the blotted DNA is exceptionally noncomplex (e.g., restricted plasmid DNA), or if the DNA is highly concentrated (e.g., colony and plaque blots; *UNIT 6.3*). Note that a membrane hybridized to a ^{35}S-labeled probe has to be dried before autoradiography, so probe stripping is not possible.

Nonradioactive probes are a more realistic option for hybridization analysis of genomic DNA and are becoming increasingly popular as the problems involved in their use are gradually ironed out. Their advantages include greater safety, the fact that large amounts of probe can be prepared in one batch and stored for years, and the rapidity of the detection protocols. Their main disadvantage is that the sensitivity of most nonradioactive detection systems is lower than that of ^{32}P autoradiography, which means that the blot and hybridization have to be carried out at maximum efficiency if a satisfactory signal is to be seen. For details on hybridization analysis with nonradioactive probes, see *UNITS 3.18 & 3.19* and Mundy et al. (1991).

Using an inert polymer to increase sensitivity. In addition to adjusting the parameters discussed above, an improvement in sensitivity can also be achieved by adding an inert polymer such as 10% (w/v) dextran sulfate (molecular weight 500,000) or 8% (w/v) PEG 6000 to the hybridization solution. Both induce an increase in hybridization signal, 10-fold with a single-stranded probe and as much as 100-fold if the probe is double-stranded (Wahl et al., 1979; Amasino, 1986). The improvement is thought to arise from formation of interlocked meshes of probe molecules, which anneal en masse at target sites. Increased hybridization signals are a major bonus in detecting single-copy sequences in complex genomes, but this must be balanced with the fact that the polymers make the hybridization solutions very viscous and difficult to handle.

Factors influencing specificity

Ensuring specificity in homologous hybridization experiments. The hybridization incubation is carried out in a high-salt solution that promotes base-pairing between probe and target sequences. In 5× SSC, the T_m for genomic DNA with a GC content of 50% is about 96°C. Hybridization is normally carried out at 68°C, so the specificity of the experiment is not determined at this stage. Specificity is the function of post-hybridization washes, the critical parameters being the ionic strength of the final wash solution and the temperature at which this wash is carried out.

The highly stringent wash conditions described in the basic and alternate protocols should destabilize all mismatched heteroduplexes, so that hybridization signals are obtained only from sequences that are perfectly homologous to the probe. For DNA and RNA probes (as opposed to oligonucleotides), problems with lack of specificity after the highly stringent wash occur only if the hybridizing sequences are very GC-rich, resulting in a relatively high T_m. If the high-stringency wash does not remove all nonspecific hybridization, temperature can be increased by a few degrees. The equations above for calculating T_m can be used as a guide for selecting the correct temperature for the final wash, but trial and error is more reliable. Note that a membrane that has been autoradiographed can be rewashed at a higher stringency and put back to expose again, the only limitation being decay of the label and the need for a longer exposure the second time.

Table 2.10.3 High-Salt Solutions Used in Hybridization Analysis

Stock solution	Composition
20× SSC	3.0 M NaCl/0.3 M trisodium citrate
20× SSPE[a]	3.6 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA, pH 7.7
Phosphate solution[b]	1 M $NaHPO_4$, pH 7.2[c]

[a]SSC may be replaced with the same concentration of SSPE in all protocols.

[b]Prehybridize and hybridize with 0.5 M $NaHPO_4$ (pH 7.2)/1 mM EDTA/7% SDS [or 50% formamide/0.25 M $NaHPO_4$ (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS]; perform moderate-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/5% SDS; perform high-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/1% SDS.

[c]Dissolve 134 g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter water, then add 4 ml 85% H_3PO_4. The resulting solution is 1 M Na^+, pH 7.2.

Designing washes for heterologous hybridization. Calculations of T_m become more critical if heterologous probing is being attempted. If the aim is to identify sequences that are merely related, not identical, to the probe (e.g., members of a multigene family, or a similar gene in a second organism), then it is useful to have an idea of the degree of mismatching that will be tolerated by a "moderate-" or "low-" stringency wash. The best way to approach this is to first establish the lowest temperature at which only homologous hybridization occurs with a particular SSC concentration. Then assume that 1% mismatching results in a 1°C decrease in the T_m (Bonner et al., 1973) and reduce the temperature of the final wash accordingly (for example, if sequences with ≥90% similarity with the probe are being sought, decrease the final wash temperature by 10°C). If the desired degree of mismatching results in a wash temperature of <45°C, then it is best to increase the SSC concentration so that a higher temperature can be used. Doubling the SSC concentration results in a ~17°C increase in T_m, so washes at 45°C in 0.1× SSC and 62°C in 0.2× SSC are roughly equivalent. Note that in these extreme cases it may also be necessary to reduce the hybridization temperature to as low as 45°C (aqueous solution) or 32°C (formamide solution).

This approach sometimes works extremely well (as shown when the heterologous targets are eventually sequenced), but the assumption that a 1% degree of mismatching reduces the T_m of a heteroduplex by 1°C is very approximate. Base composition and mismatch distribution influence the actual change in T_m, which can be anything between 0.5° and 1.5°C per 1% mismatch (Hyman et al., 1973). Unfortunately trial and error is the only alternative to the "rational" approach described here.

Other parameters relevant to hybridization analysis

Length of prehybridization and hybridization incubations. The protocols recommend prehybridization for 3 hr with nitrocellulose and 15 min for nylon membranes. Inadequate prehybridization can lead to high backgrounds, so these times should not be reduced. They can, however, be extended without problem.

Hybridizations are usually carried out overnight. This is a rather sloppy aspect of the procedure, because time can have an important influence on the result, especially if, as described above, an excess amount of a single-stranded probe is being used. The difficulties in assigning values to the parameters needed to calculate optimum hybridization time has led to the standard "overnight" incubation, which in fact is suitable for most purposes. The exception is when hybridization is being taken to its limits, for instance in detection of single-copy sequences in human DNA, when longer hybridization times (up to 24 hr) may improve sensitivity if a single-stranded probe is being used. Note that this does not apply to double-stranded probes, as gradual reannealing results in only minimal amounts of a double-stranded probe being free to hybridize after ~8 hr of incubation.

Formamide hybridization buffers. Formamide destabilizes nucleic acid duplexes, reducing the T_m by an average of 0.6°C per 1% formamide for a DNA-DNA hybrid (Meinkoth and Wahl, 1984) and rather less for a DNA-RNA hybrid (Casey and Davidson, 1977; Kafatos et al., 1979). It can be used at 50% concentration in the hybridization solution, reducing the T_m so that the incubation can be carried out at a lower temperature than needed with an aqueous solution. Originally formamide was used with nitrocellulose membranes as a means of prolonging their lifetime, as the

HYBRIDIZATION WITH RADIOACTIVE PROBES

After plaques or colonies have been transferred to nitrocellulose filters, the desired clone can be detected by its ability to hybridize to a DNA probe. This is a rapid, effective screening procedure that allows the identification of a single clone within a population of millions of other clones. The filters are hybridized with a ^{32}P-labeled nucleic acid probe, the excess and incorrectly matched probe is washed off the filter, and the filter is autoradiographed. Two features of the nucleic acid probe used for these experiments are critical to the successful screening of recombinant DNA libraries. First, the probe must hybridize only to the desired clones and not to any other clones. Thus, the nucleic acid sequence used for a probe must not contain any reiterated sequences or sequences that will hybridize to the vector. Second, the specific activity of the probe must be at least 10^7 cpm/μg. Most of the procedures for labeling DNA or copy RNA molecules are described in Chapter 3, and a support protocol is presented here that allows the 5′ end-labeling of a mixture of oligonucleotides.

The two basic protocols presented in this section describe steps required to hybridize labeled probes to recombinant DNA clones on filters. Two protocols are presented because conditions for hybridizing short oligonucleotide probes and longer nucleic acid probes to filters are different.

UNIT 6.3

Using DNA Fragments as Probes

BASIC PROTOCOL

HYBRIDIZATION IN FORMAMIDE

Bacteriophage plaques or bacterial colonies bound to a filter membrane are detected by hybridization with a radioactive probe. Hybridization proceeds on prewet filters placed in a sealable plastic bag. After hybridization the filters are removed from the sealed bag, excess probe is washed off, and the filters are autoradiographed to identify the clones that have hybridized with the probe.

Materials

Nitrocellulose membrane filters bearing plaques, colonies, or DNA (*UNITS 6.1 & 6.2*)
Hybridization solution I
Radiolabeled probe, 1 to 15 ng/ml (*UNIT 3.5*)
2 mg/ml sonicated herring sperm DNA
High-stringency wash buffer I
Low-stringency wash buffer I

Sealable bags
42°C incubator
Water bath adjusted to washing temperature (see commentary)
Glass baking dish

Additional reagents and equipment for autoradiography (*APPENDIX 3*)

Incubate filters with probe

1. Wet filters with hybridization solution I. Lay a filter membrane bearing plaques on top of 5 to 20 ml of hybridization solution I and allow solution to seep through filter. It is important to wet only one surface at a time to prevent trapping air in filter. Wet each filter in turn, producing a stack of wet filters.

   When multiple filters are to be hybridized to the same probe, no more than twenty 8.2-cm discs or ten 20 × 20 cm square filters should be placed in one stack.

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Estimate the volume of hybridization solution used to wet the filters; this is a significant fraction of the volume of the hybridization reaction.

2. Transfer the stack of wetted filters to an appropriately sized sealable bag. Add enough hybridization solution to generously cover filters and seal.

   Note the volume of hybridization solution used to cover the filters.

3. Prehybridize filters by placing the bag in a 42°C incubator for at least 1 hr.

   Some investigators omit this step.

4. While filters are prehybridizing, pipet the radioactive probe into a screw-cap tube, add 2 mg (1 ml) sonicated herring sperm DNA, and boil 10 min. Place boiled probes directly into ice to cool.

   The amount of probe used is important, and should be in the range of 1 to 15 ng/ml of hybridization reaction. The volume of the hybridization reaction can be assumed to be the amount of hybridization solution added to the filters.

5. Add 2 ml hybridization solution I to the boiled probe.

6. Remove bag containing filters from the 42°C incubator. Open bag, add probe mixture, exclude as many bubbles as possible, and reseal.

   A good way to add the radioactive probe is to take it up in a syringe with an 18-G needle and then inject it into the bag. Reseal the bag after adding probe.

7. Mix probe in the bag so that filter is evenly covered. Replace bag in the 42°C incubator and let hybridize overnight.

Wash filters to remove nonhybridized probe

8. Warm 1 liter high-stringency wash buffer I to the "washing temperature" in a water bath.

   The stability of washing temperature and salt concentrations are critical features of this experiment. See discussion in commentary.

9. Remove bag containing hybridizing filters from the 42°C incubator. Cut bag open and squeeze hybridization solution out of the bag.

   CAUTION: *Handle material carefully as it is extremely radioactive. This should be done on disposable paper bench covers.*

10. Quickly immerse the filters in 500 ml low-stringency wash buffer I at room temperature in a glass baking dish. Separate all the filters, as they may stick together during hybridization.

    The volume of the low-stringency wash buffer is not important as long as the filters are completely covered. The filters must not be allowed to dry as the radioactive probe will irreversibly bind the filters if the filters dry in contact with probe. (The type of container used to hold the filters is not important as long as it transfers heat well. Thus glass, metal, or enamel containers are better than plastic.)

    The low-stringency wash only removes nonhybridized probe formamide and hybridization solution; it does not determine the stringency of the hybridization.

11. Rinse the filters three times with 500 ml low-stringency wash buffer. Let the filters sit 10 to 15 min at room temperature in low-stringency wash buffer with each rinse.

12. Pour off the low-stringency wash buffer and pour in 500 ml high-stringency wash buffer (prewarmed to washing temperature).

13. Replace the high-stringency wash buffer with another 500 ml of high-stringency wash buffer, then place the glass dish containing the filters in incubator at wash temperature. Make sure that the temperature in the glass dish reaches the desired washing temperature by placing a thermometer directly into the bath and measuring the temperature. Usually 15 to 20 min at the desired wash temperature is sufficient to remove most of the background radioactivity.

Of course, if the glass dish is placed in a water bath, be careful that the water from the water bath does not get into the filters.

Autoradiographing filters

14. Remove filters and mount them either wet or dry on a plastic backing. If the filter(s) is to be exposed wet, then isolate it from the film by covering it with plastic wrap.

Used X-ray film provides a good form of plastic backing for filters.

15. Mark the filters with radioactive ink to assist in alignment and autoradiograph.

An easy way to apply radioactive ink is to mark adhesive-backed paper labels with radioactive ink and then attach the stickers to the plastic wrap cover.

X-ray intensifying screens greatly decrease the amount of exposure time required.

ALTERNATE PROTOCOL

HYBRIDIZATION IN AQUEOUS SOLUTION

This method differs mainly in that formamide is not used in the hybridization solution. Follow the basic protocol except use the reagents and alternate parameters listed below.

Additional Materials

Hybridization solution II
Low-stringency wash buffer II
High-stringency wash buffer II
65°C incubator

1. Prehybridize as in basic protocol except that the filters are prehybridized at 65°C using hybridization solution II.

Hybridization solution II may have to be prewarmed to solubilize the SDS.

2. Prepare probe as in step 4 of basic protocol and dilute with 2 ml of hybridization solution II.
3. Hybridize overnight as in steps 6 and 7 of basic protocol except use a hybridization temperature of 65°C.
4. Remove bag containing hybridization from the 65°C incubator. Squeeze out the hybridization solution, taking care to avoid contamination with the excess radioactive hybridization solution.
5. Immediately rinse filters twice with low-stringency wash buffer II.

It is unnecessary to maintain a given temperature for this wash; just let the filters sit in wash buffer at room temperature until ready to proceed.

6. At 65°C, proceed to wash filters with high-stringency wash buffer II. Employ multiple quick washes (5 to 8) and immerse filter in a final wash for ~20 min. Check the radioactivity of the filters with a Geiger counter and be certain that they produce a signal only a fewfold above background levels.

REAGENTS AND SOLUTIONS

High-stringency wash buffer I

0.2× SSC (*APPENDIX 2*)
0.1% sodium dodecyl sulfate (SDS)

High-stringency wash buffer II

1 mM Na_2EDTA
40 mM $NaHPO_4$, pH 7.2
1% SDS

Hybridization solution I

Mix following ingredients for range of volumes indicated (in milliliters):

Formamide	24	48	72	120	240	480
20× SSC	12	24	36	60	120	240
2 M Tris·Cl, pH 7.6	0.5	1.0	1.5	2.5	5.0	10
100× Denhardts solution	0.5	1.0	1.5	2.5	5.0	10
Deionized H_2O	2.5	5.0	7.5	12.5	25	50
50% dextran sulfate	10	20	30	50	100	200
10% SDS[a]	0.5	1	1.5	2.5	5	10
Total volume	**50**	**100**	**150**	**250**	**500**	**1000**

[a]In place of SDS, *N*-lauroylsarcosine (Sarkosyl) may be used.

Add the SDS last. The solution may be stored for prolonged periods at room temperature.

The dextran sulfate should be of high quality. Pharmacia produces acceptable-grade dextran sulfate. Recipes for SSC and Denhardt's solution are in APPENDIX 2.

Hybridization solution II

1% crystalline BSA (fraction V)
1 mM EDTA
0.5 M $NaHPO_4$, pH 7.2 (134 g $Na_2HPO_4 \cdot 7H_2O$ plus 4 ml 85% H_3PO_4/liter = 1 M $NaHPO_4$)
7% SDS

Low-stringency wash buffer I

2× SSC (*APPENDIX 2*)
0.1% SDS

Low-stringency wash buffer II

0.5% BSA (fraction V)
1 mM Na_2EDTA
40 mM $NaHPO_4$, pH 7.2
5% SDS

Sonicated herring sperm DNA, 2 mg/ml

Resuspend 1 g herring sperm DNA (Boehringer Mannheim #223636) in a convenient volume (about 50 ml of water) by sonicating briefly. The DNA is now ready to be sheared into short molecules by sonication. Place the tube containing the herring sperm DNA solution in an ice bath (the tube must be stable even if the ice begins to melt). The sonicator probe is placed in the DNA solution (without touching the bottom of the vessel). The sonicator is turned on to 50% power 20 min, or until there is a uniform and obvious decrease in viscosity. At no time should the tube containing the DNA become hot to the touch. After sonication, the DNA is diluted to a final concentration of 2 mg/ml, frozen in 50-ml aliquots, and thawed as needed.

COMMENTARY

Background Information

All hybridization methods depend upon the ability of denatured DNA to reanneal when complementary strands are present in an environment near but below their T_m (melting temperature). In a hybridization reaction involving double-stranded DNA on a filter and a single-stranded DNA probe there are three different annealing reactions occurring. First, there are the desired probe-DNA interactions which result in signal. Second, there are mismatch interactions that occur between related but nonhomologous sequences; these mismatch hybrids are the ones that must be eliminated during the washing of the filters. Non-sequence-specific interactions also occur and these result in noise. The ability to extract information from a particular hybridization experiment is a function of the signal-to-noise ratio. High background or poor specific signal can both result in uninterpretable results.

Washing nitrocellulose filters is required to remove excess radioactive probe, as well as radioactive probe that has bound to the DNA on the filter as mismatch hybrids. Temperature and salt concentration dramatically affect the maintenance of specific hybrids. Detergents and other charged species can have a profound effect upon the nonspecific binding of species that contribute to background. In this protocol, hybridization is achieved in a solution containing 50% formamide. Excess probe is rinsed away under low-stringency conditions so that further hybridization will not occur. Once the hybridization solution is rinsed away, it is possible to proceed to a high-stringency wash without fear of further hybridization. When washing is complete, the filters should produce very little "noise" when monitored with a Geiger counter. Although single-copy sequence probe normally does not produce a signal that is detectable with a Geiger counter, a probe corresponding to more abundant sequences will produce a signal that can be "heard" with a Geiger counter.

Literature Review

Hybridization to filter membranes forms a basis of recombinant DNA technology and is described in detail earlier in the manual (*UNIT 2.9*). The protocols described here vary from those used for Southern blot filter hybridization in that the volume of the hybridization is usually larger and the washing conditions are different. Dextran sulfate is an important component of the hybridization solution as it increases the rate of reassociation of the nucleic acids.

The protocols in this unit describe methods for hybridizing radioactive probes to membrane-bound plaques or colonies. These procedures for screening recombinant clones were first suggested by Grunstein and Hognes (1975) and by Benton and Davis (1977). The conditions of hybridization proposed in the basic protocol involving hybridization in formamide was originally described by Denhardt (1966) and Gillespie and Spiegelman (1965) while the alternate protocol using aqueous hybridization solution was introduced by Church and Gilbert (1984).

The method of washing filters under stringent conditions to remove background was first proposed by Southern (1975). Botchan et al. (1976) described the benefit of adding SDS to the wash solution. Jeffreys and Flavell (1977) first employed the wash conditions described in the protocols presented here.

Critical Parameters

Hybridization. Kinetically, the hybridization of DNA (or RNA) probes to filter-bound DNA is not significantly different from hybridization in solution. For single-stranded probes, the rate of hybridization follows first-order kinetics, since probe is available in excess. Under conditions of excess probe, the time for hybridization is inversely proportional to the probe concentration. For double-stranded probes the rate of hybridization displays a more complex relationship to the initial probe concentration. However, to a first approximation the initial probe concentration is inversely proportional to the rate of hybridization. To determine the actual time required for the successful hybridization of a given probe, either empirical data must be available or the following formula can be used to determine the length of time (in hours) required to achieve 50% hybridization (T_{50}):

$$1/x \times y/5 \times z/10 \times 2 = T_{50}$$

where x is the weight of probe in micrograms; y is the complexity of probe in kilobases; and z is the volume of hybridization solution in milliliters. The length of time T is given in hours. Maximum hybridization signal will be obtained if the reaction is allowed to proceed to $5 \times T_{50}$, although 1 to $2 \times T_{50}$ is often used.

It is also clear that nonspecific interactions

occur and that in any hybridization, sources of noise will be present. Therefore, from a practical standpoint one conventionally utilizes concentrations of nick-translated probe on the order of 1 to 15 ng/ml of hybridization, where the specific activity of the probe is from 5×10^7 cpm/μg to $>10^8$ cpm/μg. Too much probe in a hybridization is as bad as too little.

One important source of background hybridization to filters is due to the hybridization of the probe to vector sequences or to *E. coli* DNA. Be certain that there is no vector or *E.coli* DNA sequences in the probe. This can best be ensured by isolating the probe from one type of vector (e.g., plasmid) and screening a library made with a different type of vector (e.g., bacteriophage).

Washing temperature. Washing at low stringency is a straightforward proposition. Buffer is added at room temperature and washing proceeds at room temperature.

High-stringency wash is determined empirically. The relative homology between the probe and target sequence is a determining parameter. If the homology is 100%, a high temperature (65° to 75°C) can be used. As the homology drops, lower washing temperatures must be used. In general one starts at 37° to 40°C, raising the temperature by 3° to 5°C intervals until background is low enough not to be a major factor in the autoradiography.

The length of the probe is also important. Very short probes (<100 bp) must be washed at lower temperatures, even if the homology is 100%. Washing strategy is the same as for probes of differing homology.

Salt concentration. The lower the salt concentration, the higher the stringency. With this said, the protocols as outlined do not require adjustment of salt concentration for adjustment of stringency. Only the washing temperature is varied.

Probe. The nucleic acid probe must be of high specific activity and greater than 50 bp in length so that it can form stable hybrids.

Anticipated Results

After washing the filters the background should be barely detectable with a Geiger counter.

With a high-specific-activity probe $>5 \times 10^7$ cpm/μg and an overnight hybridization reaction with a 1-kb unique sequence probe, hybridizing bacterial colonies or bacteriophage plaques can be visualized after a 1 to 18 hr exposure.

Time Considerations

Generally hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots. However, with probes of increasing complexity longer hybridization times are required. This is preferable to increasing the probe concentration from experiment to experiment.

Autoradiography requires 1 to 18 hr.

Literature Cited

Benton, W.D. and Davis, R.W. 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. *Science* 196:180.

Botchan, M., Topp, W., and Sambrook, J. 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. *Cell* 9:269-287.

Church, G. and Gilbert, W. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 81:1991-1995.

Denhardt, D. 1966. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.

Gillespie, D. and Spiegelman, S. 1965. A quantitative assay for DNA–RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* 12:829-842.

Grunstein, M. and Hogness, D. 1975. Colony Hybridization: A method for the isolating of cloned DNA's that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* 72:3961.

Jeffreys, A.J. and Flavell, R.J. 1977. A physical map of the DNA region flanking the rabbit β globin gene. *Cell* 12:429-439.

Southern, E.M. 1975. Detection of specific sequence among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

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